

APPLICATIONS OF ADENINE NUCLEOTIDE MEASUREMENTS IN OCEANOGRAPHY

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INTRODUCTION

All our oceanographic studies involving measurement of ATP, ADP, or AMP are based on the following two premises.

- All live microbial cells in our samples contain a relatively uniform amount of ATP per unit cell volume. As dead cells and detrital material do not contain any significant amount of ATP, the measurement of ATP permits estimation of total microbial biomass in terms of weight or cellular organic carbon.
- The cellular concentrations of adenine nucleotides are important in regulation of metabolic rates. The relative concentrations of these nucleotides thus can yield information on the physiological state of the cells.

Before discussion of our specific uses of data on the distribution of these nucleotides, we wish to briefly outline (1) the methodology involved in these measurements and (2) our data to support the premise that ATP concentrations in microbial cells can be extrapolated to biomass parameters.

METHODOLOGY

Sampling

In most of our work, the concentration of cells is so low that we must concentrate the cells prior to killing and extraction for ATP. Our sampling devices range from small sterile units (for example, Niskin disposable bags) to large 200-l Van Dorn type samplers which are scrubbed with alcohol just prior to use. The interval between collection of the sample and killing of cells is kept as short as possible to minimize any changes in cellular ATP levels due to changes in pressure, temperature, or light conditions.

Killing of Cells and Extraction of Nucleotides

Cells are concentrated by filtration of the sample through microfine glass fiber filters (Reeve-Angel, No. 984H) which are then quickly immersed in TRIS buffer (0.02 M, pH 7.7) at 373 K (100° C). It is important to kill the cells and to stop all enzymatic activity as quickly as possible. The turnover time of ATP is very rapid (from about 1 s to 1 min) in most cells, and a delay in killing of the cells or a slow killing procedure can result in significant changes in ATP levels. After boiling for 4 to 5 min, the samples are stored at 253 K (-20° C) until time of analysis.

Measurement of ATP

During the past nine years, we have developed the necessary instrumentation to give us the speed, sensitivity, and flexibility that are required in our type of work. In most of our studies we measure ATP in the sample by integration of the light flux for a 1-min time period (figure 1). One important advantage of this method is that it permits one to obtain complete mixing of the sample with enzyme preparation during the 15-s delay period. It also eliminates the artifacts often associated with increased light levels due to agitation of the enzyme preparation when it is mixed with the sample. The magnitude of this latter problem varies considerably with different enzyme preparations.

For some research problems, the use of peak-height analysis offers some advantages (for example, time required per assay in analyses for ADP and AMP when enzymes other than luciferase are reacting with ATP) over the light-integration methods. For such studies we use another machine (JRB ATP Photometer) which displays either the integrated light flux or the peak-height value directly on a digital readout. The sensitivity of these photometers with partially purified enzyme is about 10^{-16} moles ATP. For a more detailed description of the methodology, see Holm-Hansen (1973).

ATP CONCENTRATIONS IN MICROORGANISMS

For our purposes the most useful parameter to use for biomass estimation is total cellular organic carbon. During the past nine years, we have studied a great variety of marine and freshwater microbial cells to obtain data on the ratio of cell carbon to ATP, and how this ratio can be altered by environmental stresses. The carbon per ATP ratio we use in our work is 250, which is based on the following types of data.

Figure 2 shows the relationship between cellular organic carbon and ATP in 30 species of unicellular algae representing 7 phyla. Details on these cultures have been described by Holm-Hansen (1970a).

Hamilton and Holm-Hansen (1967) used both batch and chemostat cultures of marine bacteria and measured ATP, total cell count, viable cell count, and

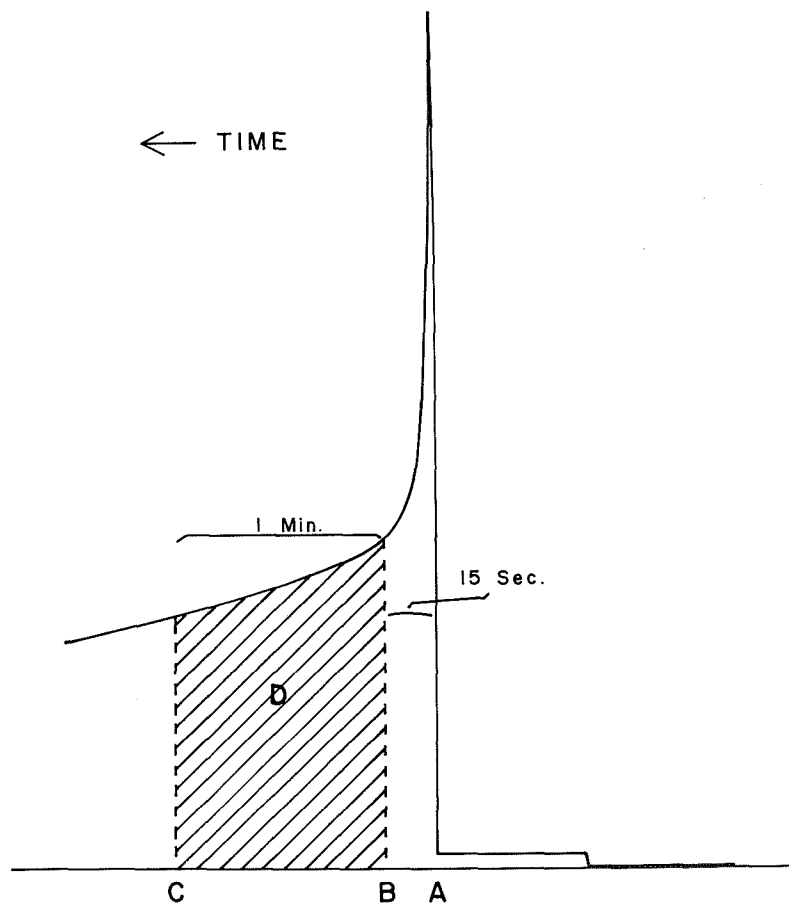


Figure 1. Light emission curve when a sample containing ATP is injected into the luciferin-luciferase enzyme preparation: A—time of injection of sample; B—end of 15-s mixing period; C—end of 1.0-min light-integration period; D—area electronically integrated (from Holm-Hansen, 1973).

cellular organic carbon. These data showed that ATP was a measure of the concentration of viable cells in the suspension and that the ratio of carbon to ATP averaged close to the above value of 250.

We have also made numerous measurements of ATP on natural phytoplankton populations, either with or without nutrient enrichment (Strickland et al., 1969; Eppley et al., 1971, Holm-Hansen, 1969). In these studies we have also measured total particulate organic carbon in addition to estimating phytoplankton-carbon by calculations based on cell counts and cell volumes as measured with an inverted microscope. In all this work, the carbon as

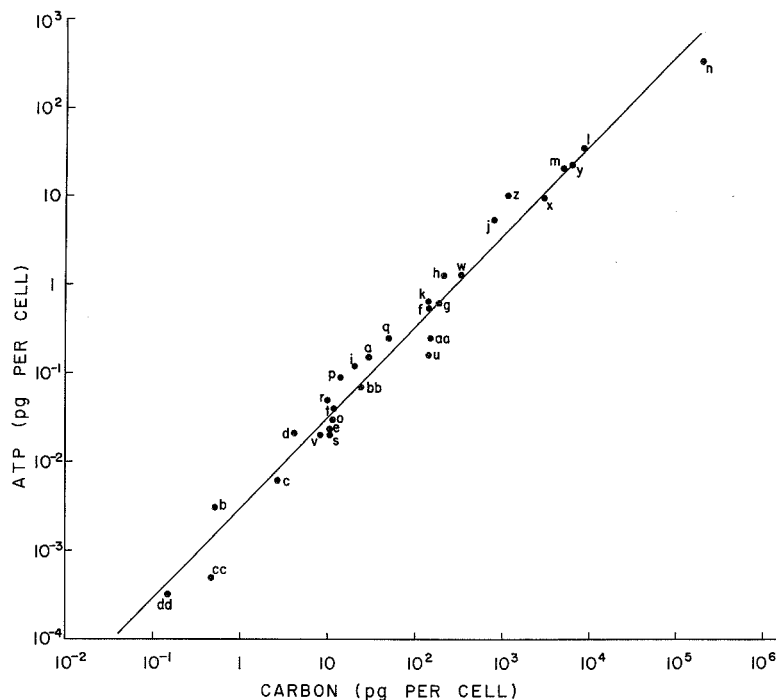


Figure 2. Cellular contents of ATP in 30 algal cultures as a function of the organic carbon per cell (from Holm-Hansen, 1970a).

estimated by ATP measurement was close to that estimated by direct floristic analyses, and was a realistic fraction of the total particulate organic carbon (ranging from about 20 percent to close to 100 percent).

As cells are taken from many depths in the water column, they will be exposed to varying light conditions during subsampling and filtration. It was therefore of importance to our field work to ascertain what changes in light conditions will do to cellular ATP levels. Holm-Hansen (1973) has shown that ATP levels in phytoplankton are maintained at fairly uniform concentrations during light/dark periods. There are transient changes in ATP levels when light conditions are suddenly changed, but the levels are reestablished within a period of 1 to 3 min.

Both microzooplankton (flagellated or ciliated protozoans) and macrozooplankton (Crustacea) also have ATP contents very close to 0.4 percent of the total cellular organic carbon. Some data for *Calanus* species are included in the paper by Holm-Hansen (1973).

During the past year, Thiel and Holm-Hansen have been studying the ATP content of various benthic animals, from both littoral and deep (1200 m) sediments. Our samples have included representatives of the Annelida,

Platyhelminthes, Aschelminthes, and Arthropoda (Crustacea). There is considerably more spread in the C/ATP ratio in these animal phyla, as the ratio in some polychaetes has been close to 100, while in some amphipods it is close to 1000. These latter individuals, however, are large (2 to 3 mg dry weight) and the exoskeleton probably accounts for close to 75 percent of the total cellular organic carbon. Most of our data on these diverse animals show carbon per ATP (C/ATP) ratios between 200 to 300. We therefore believe that the relationship of cell carbon to ATP as used for all our phytoplankton work will also be applicable to bottom-dwelling metazoans.

APPLICATIONS OF NUCLEOTIDE ANALYSES

Field Applications

One of the primary applications for which we have used ATP determinations involves questions concerning the distribution of microbial cells throughout the entire water column. The primary input of reduced carbon into the marine food chain is via phytoplankton photosynthesis, which is limited to the upper 150 m or less of the water column. We have considerable data on the distribution of larger zooplankton and fish throughout the water as well as on the benthic organisms, but we do not know the source of food necessary to support these populations. Two possibilities which are often discussed in this context are: (a) that there is an active transfer of particulate carbon from the euphotic zone throughout the entire water column by migrating zooplankton populations (Vinogradov, 1962a), and (b) that there is a significant population of heterotrophically growing cells in deep water which are grazed by filter-feeding organisms (Holm-Hansen, 1970b).

Until the introduction of ATP analyses to oceanographic work (Holm-Hansen and Booth, 1966), microbial biomass estimates were obtained either by agar-plating techniques or by direct microscopic examinations. Both of these methods have serious limitations, especially when working with deep samples (Jannasch and Jones, 1959). Figure 3 shows the microbial biomass estimated by ATP analyses to a depth of 4300 m at a station in the north Pacific. Microbial biomass decreases from 23 μg carbon in the euphotic zone (where the total particulate organic carbon was about 30 $\mu\text{g/l}$) to values of about 0.1 $\mu\text{g C/l}$ (total particulate organic carbon (POC) being about 3 to 5 $\mu\text{g C/l}$). Also shown on figure 3 are the data which Fournier (1971) obtained for total biomass of yellow-green cells from the same water samples as used for our ATP analyses. The significance of these pigmented cells in deep water is not known, although Fournier (1973) has described data which he interprets as showing that these cells may be important in deep-sea food chains. It should be noted that the biomass of microbial cells as indicated by ATP analyses is in the order of 10 times the biomass of zooplankton from similar depths as described by Vinogradov (1962b).

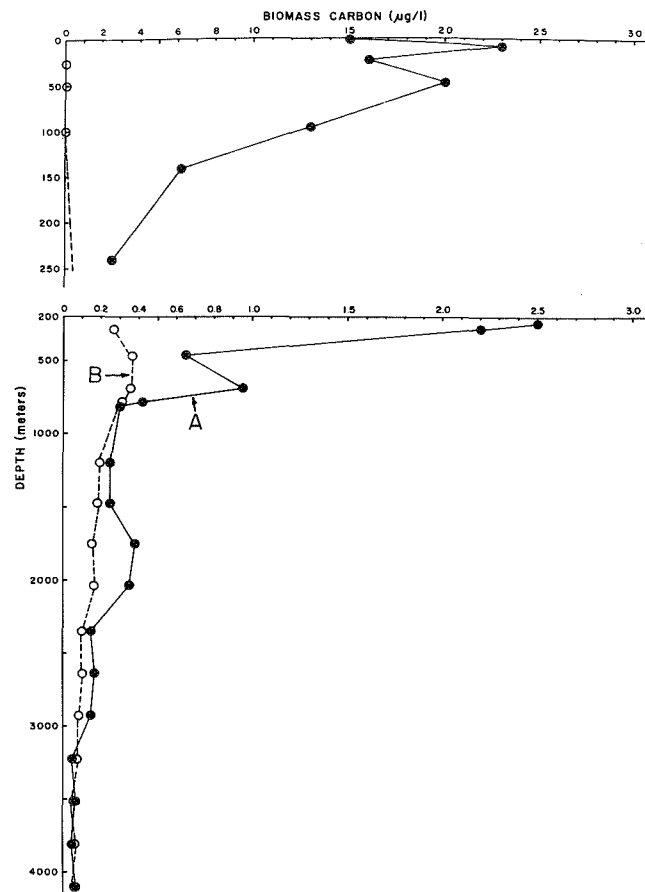


Figure 3. The distribution of microbial biomass-carbon as determined from ATP measurements (line A) and the calculated biomass-carbon contained in olive-green cells (line B) from Station Gollum north of Oahu (station position: $22^{\circ}10'N$, $158^{\circ}00'W$). The data for the olive-green cells have been taken from the paper by Fournier (1971) (from Holm-Hansen and Paerl, 1972).

The same types of problems as discussed above are also of much interest in regard to organisms living in or on bottom sediments. We are now applying ATP methodology to estimate microbial biomass in marine sediment profiles. This work is part of a larger program directed by Dr. Hessler (Scripps), which is concerned with the distribution and activity of both microbial and metazoan species in deep sea sediments.

In addition to merely assessing the standing crop of microbial cells, we have also used ATP determinations in various studies concerned with the metabolic activity of microbial cells. Two such examples are described below.

Figure 4 shows the distribution of ATP in the extremely oligotrophic waters of the north Pacific gyre region. The general characteristics of the water

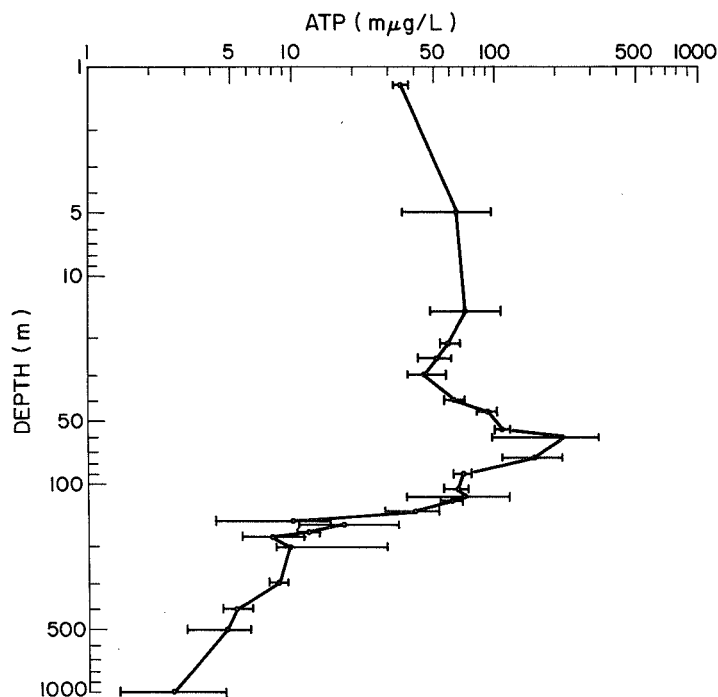


Figure 4. Vertical distribution of ATP in the north Pacific gyre.
Station position: 28°00'N, 155°00'W.

column at this location have been described by Venrick et al., (1973) and Eppley et al. (1973). The phytoplankton biomass is low in the upper 50 m and generally reaches a maximum at about 100 to 140 m. Nutrients are very low in the upper 100 m, with nitrate and phosphate generally being close to or below the detection limits of our methods (about 0.02 μ M) and silicate being about 1.0 μ M. The 1 percent light levels (the conventional compensation depth for algal photosynthesis) is usually between 80 and 140 m. The productivity profile, however, usually shows a maximum in the upper 50 m, with decreasing rates below that depth. Phytoplankton productivity in these waters thus will be nutrient-limited in the upper portion of the euphotic zone and light-limited in the lower portions. The rate of input of inorganic nutrients into the euphotic zone will therefore be one of the prime factors influencing the rate of primary production. It can be seen from figure 4 that there is a substantial microbial population between 100 and 1000 m, and it can be assumed that these cells are dependent mostly upon heterotrophic processes for their carbon and energy sources. These microbial populations are likely to be of considerable importance in regard to regeneration of nutrients which are essential for continued phytoplankton productivity.

Figure 5 shows a profile for ATP and dissolved oxygen concentrations from the surface to 7200 m at a station in the Aleutian Trench. The surface water at this station was cold (280.9 K (7.8° C)) and nutrient-rich (about 15 μ M silicate and 1.0 μ M phosphate). In contrast to the data discussed above for tropical waters, the microbial biomass was greatest in surface waters and decreased rapidly with depth. The oxygen curve shows a marked minimum at about 900 m. The origin of such oxygen minima in ocean water is not well understood, although the hypotheses of intrusion of low-oxygen water (Menzel, 1970) and in situ oxygen consumption (Craig, 1971) both have considerable support. The microbial biomass indicated by the ATP data in figure 5 can be expected to lower oxygen concentrations by their respiratory requirements. On the basis of extensive respiratory data in the literature we have extrapolated ATP-biomass data into respiratory rates, with corrections for in situ temperatures. Such respiratory estimates compared quite well with estimates based on oxygen electrode measurements and on electron transport system (ETS) activity (Hobbie et al., 1972). Packard et al. (1971) have estimated that respiration in deep water (5000 m) is in the range of 1 to 5 μ l O₂/liter/year, which is about the same range as suggested by calculations based on ATP measurements.

A particularly useful application of ATP assays is in conjunction with studies on the effects of outfalls (sewer, nuclear plant, and so forth) in coastal waters or on the effects on benthic life when waste products are dumped in deep water. The increase of microbial biomass in the vicinity of sewer outfalls has been documented by Eppley et al. (1972). Similar studies on the biomass of microbial cells in sediments would be of considerable interest, as many pollutants will have limited solubility in water and will concentrate in the sediments.

Some pollutants apparently can act either as killing agents or as growth-inhibiting agents. Fitzgerald and Faust (1963), for instance, have shown that Cu displays either algicidal or algistatic properties on various species of algae. ATP measurements can be used to differentiate between dead cells and such inhibitor-arrested cells. We are currently using this technique in our controlled ecosystem pollution experiment (CEPEX) program, in which we are examining the effects of various pollutants on natural phytoplankton populations.

Laboratory Studies

One of our main projects is the study of the factors which control or limit phytoplankton growth in the sea. Reference to figure 6 will indicate the nature of one problem in interpretation of our ¹⁴C-uptake studies. In primary production experiments using ¹⁴C-bicarbonate, the incubation period normally used ranges from a few hours to one day. When cells are very stressed by nutrient deficiency, however, there can be a 1- to 2-day lag after introduction of the limiting nutrient before reduction of CO₂ occurs at any

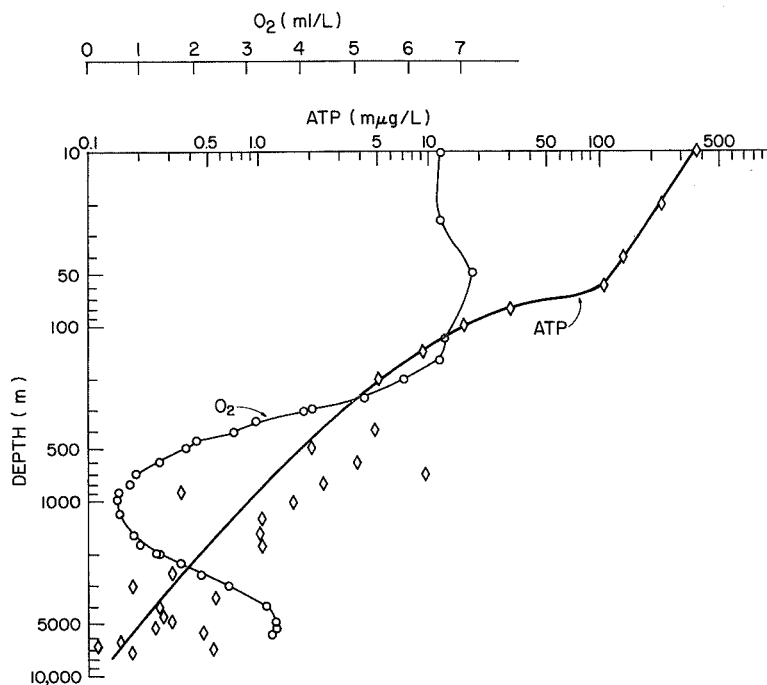


Figure 5. Vertical distribution of ATP and dissolved O₂ in the Aleutian Trench. Station position: 50°53'N, 177°31'W.

appreciable rate. There is, however, a rapid response of cellular ATP levels upon addition of the nutrient (see inset of figure 6). The chemical composition of cells during starvation and after recovery can be very different. After addition of phosphorus to P-deficient cells, for instance, the C/P ratios may change from 250 to 5 and the C/ATP ratio from 2000 to 200. Such studies on the biochemical events occurring during the period when cells are adjusting to nutrient addition are useful in conjunction with our biomass estimations based on ATP concentrations and with assessment of the nutritional status of phytoplankton populations in nature.

Most blue-green algae are obligate photoautotrophs and cannot grow heterotrophically in the dark. Smith et al. (1967) have suggested that the reason for this is that these algae lack the complete complement of tricarboxylic acid (TCA) cycle enzymes and hence are incapable of oxidative phosphorylation. They would thus depend solely upon photophosphorylation for their ATP production. Our data on ATP levels in the blue-green alga *Nostoc muscorum*, however, demonstrate that this hypothesis of Smith et al. is not correct. As in all other algae investigated, ATP levels decrease rapidly when transferred from light to dark, but within a few minutes oxidative phosphorylation restores the ATP levels to the original light level.

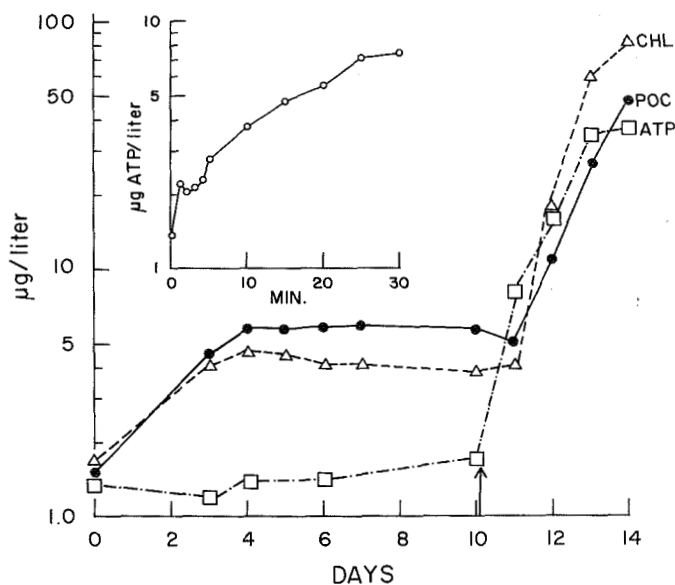


Figure 6. The response of ATP, photosynthesis (total particulate organic carbon), and chlorophyll in *Monochrysis lutheri* during phosphorus deficiency (days 1 to 10) and after addition of orthophosphate at time indicated by the arrow. ATP, $\mu\text{g/l}$; chlorophyll, $\mu\text{g} \times 10^{-1}/\text{l}$; POC, $\mu\text{g} \times 10^{-3}/\text{l}$. Inset shows ATP response from 1 to 30 minutes in a separate experiment when phosphate was added to deficient cells at time 0.

Silicon is an essential mineral element for most diatoms. When a culture of *Navicula pelliculosa* is silicon-deficient, mitosis occurs without concomitant cytokinesis (Coombs et al., 1967a). A population of binucleated cells thus results, which remains in this condition until addition of silicon. When silicon is introduced, there are rapid changes in respiration, photosynthesis, and assimilation of silicon (Coombs et al., 1967b). Transient changes in ATP levels have been followed throughout this sequence of events in order to see if the uptake of silicon is an active uptake mechanism which requires ATP. The results of these investigations have been described by Coombs et al. (1967a).

As we have seen from the other papers in this symposium, the energy charge of cells as described by Atkinson (1969) does seem to be correlated with viability and growth rate of cells. We have therefore been investigating the relative concentrations of ATP, ADP, and AMP in laboratory cultures of stressed cells discussed above. Under extreme nitrogen or phosphorus nutrient deficiency, the ratio of C/ATP increases significantly, which would result in an error in biomass estimations which assume a constant ratio of C/ATP.

We are hopeful that in such situations, measurement of all three nucleotides will be more useful than measurement of ATP alone.

In an earlier section we discussed estimation of microbial biomass in deep water and in sediments. In addition to standing crop estimates, we also need information on the metabolic activity of these cells, which requires use of other techniques such as heterotrophic uptake studies with radio-labeled substrates. We are now also examining the energy charge of these cells living in deep water in the hope that these data will be informative in regard to the energetic state of the cells.

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